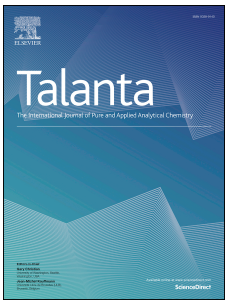
Journal Pre-proof



Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control

Florian Weber, Lisa Rahnfeld, Paola Luciani

PII: S0039-9140(20)30611-1

DOI: https://doi.org/10.1016/j.talanta.2020.121320

Reference: TAL 121320

To appear in: *Talanta*

Received Date: 25 April 2020

Revised Date: 19 June 2020

Accepted Date: 22 June 2020

Please cite this article as: F. Weber, L. Rahnfeld, P. Luciani, Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control, *Talanta*, https://doi.org/10.1016/j.talanta.2020.121320.

CRediT author statement

Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control

Florian Weber[‡], Lisa Rahnfeld[‡], Paola Luciani*

Pharmaceutical Technology Laboratory, Department of Chemistry and Biochemistry, University of Bern, Switzerland

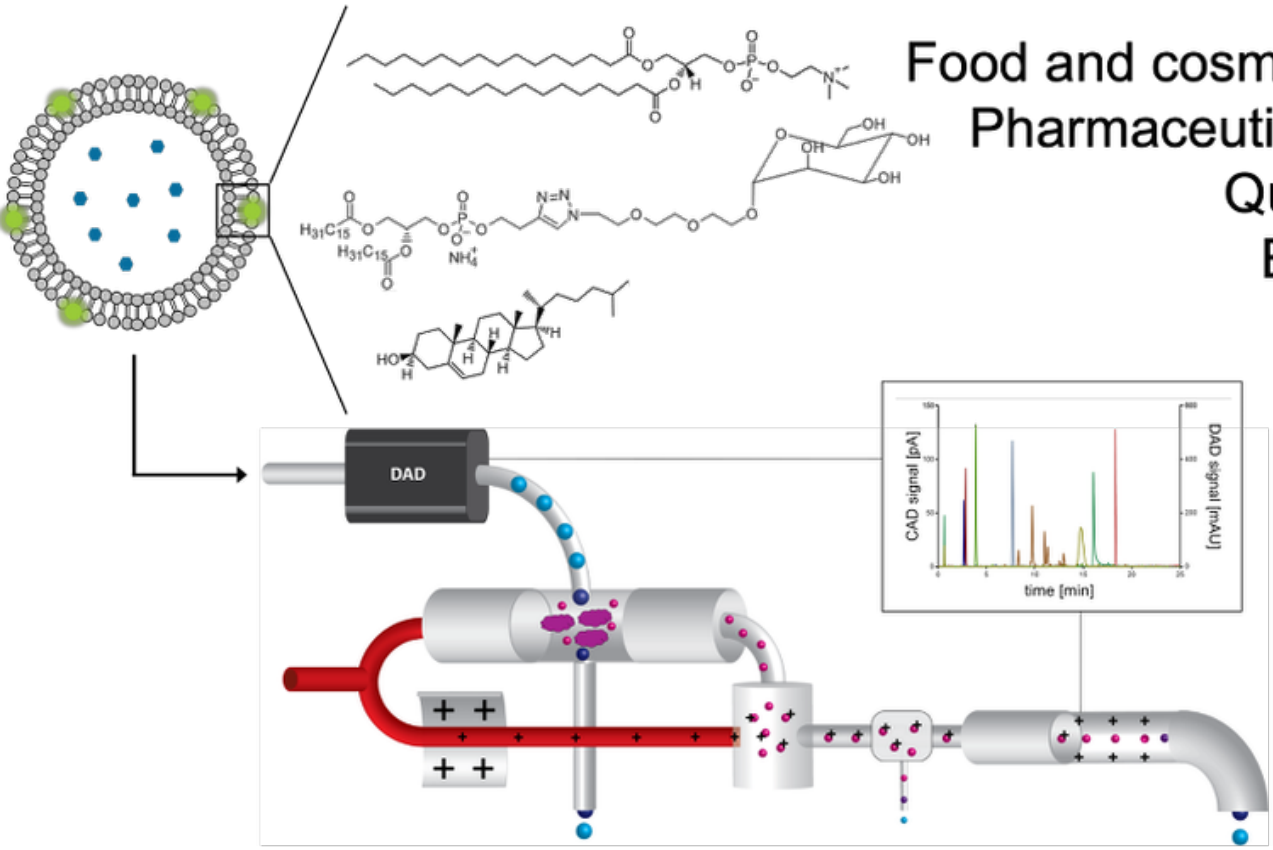
[‡] contributed equally

* corresponding author

Florian Weber: Conceptualization, Data curation, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization.

Lisa Rahnfeld: Conceptualization, Data curation, Validation, Investigation, Writing - original draft, Visualization.

Paola Luciani: Conceptualization, Writing - review & editing, Supervision, Visualization, Project administration, Funding acquisition.



Food and cosmetic industry
Pharmaceutical research
Quality control
Biochemistry

Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control

Florian Weber[‡], Lisa Rahnfeld[‡], Paola Luciani[c]*

Pharmaceutical Technology Research Group, Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland,

[‡] contributed equally

* corresponding author. Email address: paola.luciani@dcb.unibe.ch, phone: +41 31 631 4160

Abstract

Phospholipids and their derivatives represent a broad range of multifunctional substances used as excipients or active ingredients by different industries due to their natural origin and unique properties. A fast and reliable quantification as well as comprehensive stability evaluation are of major importance in the process of development and quality control of lipid-based systems. Therefore, the present study is focused on the development and validation of a rapid ultra-high performance liquid chromatography – charged aerosol detector based (UHPLC-CAD) method for simultaneous detection of a multitude of natural and synthetic lipids, (charged) phospholipids, lipophilic fluorescent markers and their possible degradation products. Twenty-two compounds were characterized by a strong linear response of the detector ($R^2 > 0.97$). Moreover, remarkable limits of detection (≤ 10 µg/mL) and limits of quantification (≤ 25 µg/mL) associated with a consistent reproducibility were achieved for all tested molecules. The performance of the analytical method was demonstrated by analyzing the lipid composition (after differ-

ent production stages and photodegradation) of both bupivacaine loaded liposomes and a Doxil®-like formulation. The newly developed method combines a rapid, comprehensive, and efficient quantification with minimal economic effort and ecologic consequences, meeting the requirements of modern analytical processes and offering a broad range of possible applications in various industrial sectors and scientific laboratories.

Keywords: UHPLC-CAD method, phospholipid detection, phospholipid quantification, liposomes, charged aerosol detector

1. Introduction

Phospholipids and their derivatives represent a broad range of multifunctional substances used as excipients or active ingredients by different manufacturing branches due to their unique properties and their natural origin. They are characterized by a hydrophilic headgroup composed of a phosphate-coupled choline, serine, glycerol or ethanolamine linked to a glycerol backbone esterified with up to two hydrophobic fatty acid chains leading to the molecules amphiphilic characteristics [1,2]. Phospholipids are described as main component of cellular membranes and are therefore characterized by a practically nonexistent toxicity making them ideal components for food and cosmetic industry [3,4]. Indeed, phospholipids find their technological application as emulsifiers, antioxidants, surface-active compounds, wetting agents or skin penetration enhancer [5,6]. Furthermore, following their pivotal physiological function, phospholipids are used to produce complex colloidal systems like liposomes, solid lipid nanoparticles and artificial cell-like vesicles to generate versatile biomaterials and smart therapeutics for controlled drug delivery [4,7,8]. Being the first nano-sized drug carrier approved in 1995 by the Food and Drug Administration (FDA), liposomes underwent a great development as drug delivery system and continuous efforts have been made to build on their clinical success. Nowadays, a vast indication range is

covered by liposomal formulations, such as management of cancer, pain therapy, vaccination and treatment of severe fungal infections [9]. In the last decades, the major focus in liposome technology shifted from a plain quest for improved bioavailability of poorly water-soluble drugs to a multifaceted approach aimed either at enhancing the therapeutic efficacy by a specific targeting or by a controlled and prolonged drug release. To meet this goal a large variety of (semi-)synthetic components such as charged phospholipids, polyethylenglycol (PEG-) derivates, surfactants and other specific long-chained lipids like non-exchangeable membrane staining indocarbocyanine dyes (DiO, DiD; *vide infra*) could be introduced in the formulation, resulting in very complex compositions [10,11]. In all the above-mentioned areas, qualitative and quantitative analysis of the used phospholipids is of paramount interest for the development of new products, during the continuous monitoring of the production process or in final quality controls. The oldest methodology to quantify (phospho)lipids in liposomal formulations is based on the use of color reagents able to form complexes, then analyzed spectrophotometrically. The most established colorimetric methods for phospholipid analysis are the Bartlett assay and Stewart assay [12,13]. While the Bartlett assay is based on the analysis of inorganic phosphate occurring after the oxidation of phospholipids with perchloric acid and the subsequent reaction with ammonium molybdate, the Stewart assay enables to detect only organic phosphate after complexing phospholipids with ammonium ferrothiocyanate in chloroform. Both methods require different preparation steps and are therefore prone to an inter-user variability and a high error rate. Furthermore, only the total phospholipid concentration could be determined; molar ratios of multicomponent mixtures and their specific degradation products as well as other lipids than phospholipids cannot be analyzed with these methods making them ineffectual for routine quality control [14,15]. An instrumental approach able to evaluate a majority of the above-mentioned parameters is the analysis via gas chromatography (GC). This technique has the advantage of providing information about the exact fatty acid composition of single substances. However, a GC-based assay requires the derivatization of phospholipids to vaporable substances via acid-catalyzed

methanolysis to form fatty acid methyl esters (FAMEs) representing a critical, error-prone step [16,17]. Furthermore, being a definite differentiation of the individual element of a multicomponent liposomal formulation by means of GC not achievable, this technique results unsuitable for quality control and process monitoring.

A possible method for an efficient quantification of different components, impurities and degradation products is a gradient-based high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC) [18]. Regrettably, the absence of chromophores in lipids encumbers the analysis of all components with commonly available UV-diode array detectors (UV-DAD) and fluorescence detectors [19]. The most sophisticated method to identify different headgroups and acyl chains, and the resulting differences in polarity and charge, is detecting phospholipids by means of a mass spectrometer (MS) coupled to an UHPLC. This method also offers the opportunity of analyzing different substances eluting with the same retention time [20]. However, the costs of an MS detector compared to conventional ones, the need for experienced users, and the specific maintenance required disqualifies this technology from becoming the ideal routine (phospho)lipid analytics in conventional quality control [20]. In the event substances lack a chromophore general-purpose detectors such as refractive index detectors (RID), evaporative light scattering detectors (ELSD), condensation nucleation light scattering detectors (CNLSD) or charged aerosol detectors (CAD) could be employed [21–23]. Despite its attractively low costs and uncomplicated mode of operation, a gradient-based separation induces a permanent change in the refractive index making the detection with RID impossible [24]. In contrast to the RID, CAD, CNLSD and ELSD enable the detection even upon gradient-based separation. All three detectors can identify non-volatile substances relative to their volatile mobile phase [25,26]. Among them, the CAD offers the ability to detect semi-volatile substances like short-chained lipid components [15,27].

In a typical CAD setup, schematically shown in Figure 1, the mobile phase containing the separated analytes is nebulized by means of nitrogen to form small aerosol droplets in the CAD after passing the DAD.

After removing aerosol droplets too large for evaporation, the eluent and all volatile substances are evaporated in a tempered nebulizing chamber to form dried particles of the analyte. These particles are then combined with charged nitrogen generated by means of a corona discharge needle in the ionizer. After passing an ion trap that removes excess ions and high mobility particles, the aggregate charge of the remaining aerosol particles is measured by an electrometer which generates the signal [27].

The aim of the present study is the development and validation of a rapid UHPLC-DAD-CAD-based method for simultaneous detection of a wide range of natural and synthetic lipids, phospholipids, their degradation products and non-exchangeable fluorescent lipid markers used in the development and preparation of liposomal formulations. Furthermore, a liposomal bupivacaine formulation (BUP-liposomes) was then used to demonstrate the analytical method either for quality control or for the quantification of degradation. These BUP-liposomes represent a novel strategy for the development of drug delivery systems for sustained drug release and are based on negatively charged phospholipids (NCP) in the liposomal membrane using an *in situ* depot formation by controlled aggregation with divalent cations [28]. The encapsulation of a local anesthetic like bupivacaine could trigger a long-lasting pain reduction. Additionally, a liposomal formulation mirroring the market product Doxil was analyzed with the developed method as proof-of-principle for commercial liposome compositions. Although the applicability of the new UHPLC-CAD method is demonstrated for liposomal formulations only, the method is universal and could be applied in any possible field of interest working with phospholipids.

2. Materials and Methods

2.1 Material. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-glycero-

3-phosphocholine (POPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate sodium salt (DPPA), 1,2-distearoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt (DSPG), 1,2-dioleoyl-*sn*-glycero-3-phosphate sodium salt (DOPA), N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (sodium salt) (DSPE-mPEG-2000), hydrogenated L-α-phosphatidylcholine from soy (HSPC), L-α-phosphatidylcholine from soy (SPC), L-α-phosphatidylcholine from egg (EPC) and sphingomyelin from egg (ESM) were kindly gifted by Lipoid (Ludwigshafen, Germany). 1,2-dipalmitoyl-sn-glycero-3-phospho((ethyl-1',2',3'-triazole)triethyleneglycolmannose) (ammonium salt) (DPPA-PEG2-Mannose), 1,2-dioctadecanoyl-sn-glycero-3-phosphate (sodium salt) (1,2-dioctadecanoyl-sn-glycero-3-phosphate (sodium salt) (DSPA), 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DPPS), 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), L-alpha-phosphatidylserine (brain, porcine) (sodium salt) (BrainPS) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS) were purchased from Avanti Polar Lipids (Alabaster, USA). 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate Salt (DiD) were bought from Thermo Fisher Scientific (Waltham, MA, USA). Cholesterol (CHOL), palmitic acid, oleic acid, stearic acid and lidocaine hydrochloride were obtained from Merck (Darmstadt, Germany), trifluoracetic acid (TFA) and sodium chloride (NaCl) were obtained from Carl Roth (Karlsruhe, Germany). Bupivacaine (BUP) hydrochloride was obtained from Fagron (Glinde, Germany). All solvents used for the mobile phase were obtained by Fisher Scientific (Schwerte, Germany).

2.2 UHPLC-DAD-CAD assay. The quantification of all samples was performed by means of an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Basel, Switzerland). The instrument was equipped with a quaternary pump (LPG-3400SD), an autosampler (WPS-3000), a thermostatted column compartment (TCC-3000), a DAD (DAD-3000) and a CAD (Corona Veo RS). Chromeleon 7.2 software was used for data evaluation and instrument control. A Hypersil Gold™ column (C18, 150 x 2.1 mm) with a particle size of

1.9 µm and a pore size of 175 Å (Thermo Fisher Scientific, Basel, Switzerland) was used at a temperature of 50 °C as stationary phase. During the time of analysis, samples were kept refrigerated at 6 °C in the autosampler. The injection volume was 5 µL and the flow rate was set at 0.7 mL min^{-1}. Three different eluents, eluent A: acetonitrile +0.2% v/v trifluoracetic acid (TFA), eluent B: methanol (MeOH) +0.2% v/v TFA and eluent C: ultrapure water +0.2% v/v TFA were used to create a linear gradient in the mobile phase. The analysis started with 35% eluent A, 50% eluent B and 15% eluent C at time point 0 min. After 20 min, the mobile phase was composed of 5% eluent A and 95% eluent B and kept constant for 1.5 min. At 23 min, the composition of eluents was brought back to the initial state and the column was equilibrated for additional 3 min. For the Corona Veo RS, the gas evaporation temperature was adjusted to 45 °C and the response rate was set to 100 pA.

2.3 Quantification of phospholipids. Prior to injection, lipid stock solutions used for the calibration were prepared in MeOH. After diluting the stock solutions to appropriate concentrations, samples were then additionally diluted with the internal standard (IS, palmitic acid in MeOH 200 µg mL^{-1}) in a ratio of 1:2 to achieve a concentration of 100 µg mL^{-1} of IS in the sample. If not stated otherwise, the calibration for synthetic (phospho)lipids was performed at concentrations between 0.1 and 100 µg mL^{-1}, while the range for natural phospholipids was 0.1 - 1000 µg mL^{-1}.

2.4 Method validation. The validation of this HPLC method was done in accordance to ICH-Q2A and Q2B guidelines [29,30]. Linearity was assessed with a regression line by the method of least squares (GraphPad Prism 8.0). The limit of detection (LOD) and the limit of quantification (LOQ) were determined using the signal-to-noise ratio (S/N ratio) calculated by the Chromeleon software. The LOD was set to a S/N ratio larger than 2 and the LOQ to a S/N ratio larger than 10. The reproducibility of the calibration method was done by an intra-laboratory repetition of the calibration of DPPC, DOPC and POPC. The accuracy of the method was checked by measuring solutions of known concentrations (100, 50 and 25 µg mL^{-1}) of DPPC, DOPC and POPC and expressing the deviation in percentage from the anticipated concentra-

tion. The robustness of the method was assessed by implementing minor alterations in column oven temperature (T = ±2 K), variations in the flow rate (V_f = 0.675 mL min^{-1} and V_f = 0.65 mL min^{-1} instead of V_f = 0.70 mL min^{-1}) and CAD gas evaporation temperature (T = ±5 K) of the analytical method.

2.5 Production of liposomes and encapsulation of bupivacaine. Two different formulations of liposomes were produced: (i) liposomes containing DSPG, CHOL and DPPC (25:30:45 mol%) with a total lipid concentration of 50 mM and (ii) liposomes containing HSPC, CHOL and DSPE-PEG-2000 (56.5:38.2:5.3 mol%) with a total lipid concentration of 21.6 mM according to the composition of Doxil using film hydration method [31–33]. Briefly, after the preparation of stock solutions with appropriate organic solvents, lipids were mixed and the lipid film was produced by nitrogen stream and subsequent vacuum overnight. Large unilamellar vesicles were formed by hydrating the lipid film with an appropriate buffer. After undergoing six freeze-thaw-cycles, liposomes were extruded through a 200 nm- (formulation i) or a 100 nm-pore size (formulation ii) polycarbonate membrane (Whatman, GE Healthcare, Illinois, USA) by means of a Lipex Extruder (Evonik Industries AG, Essen, Germany). The drug BUP, a weak base with a reported pKa of 8.1, was then remotely loaded into liposome formulation (i) using an active encapsulation method based on a transmembrane ammonium gradient [34,35]. The transmembrane ammonium gradient was created by size exclusion chromatography (SEC) with Sephadex G50 (GE Healthcare, Illinois, USA) equilibrated with 150 mM NaCl solution in ultrapure water using a centrifuged-based spin protocol (3 min at 1000 g) to avoid further dilution of the sample. Subsequently, liposomes were incubated with BUP at an initial molar drug-to-lipid ratio (D/L) of 5 for 30 min at 70 °C. The non-encapsulated BUP was removed by an additional SEC step. The phospholipid amount at different stages of the production process was quantified by HPLC as well as the initial and final BUP concentration. Afterwards, the encapsulated BUP and the final D/L ratio could be calculated.

2.6 Quantification of bupivacaine by HPLC. For the quantification of BUP the above-mentioned HPLC system was used. The stationary phase, a Nucleosil C18 EC (250 mm x 4 mm) with a particle size of 5 µm

(Macherey-Nagel, Düren, Germany), was kept at 50 °C. A linear gradient method with a flow rate of 1 mL min^{-1} with eluent A: ACN with 0.1% v/v TFA and eluent B: ultrapure water with 0.1% v/v TFA was used. The method started with 25% of eluent A changing to an equal mixture after 15 min. Afterwards, the composition was changed to 99% eluent A. After reaching the initial composition of the mobile phase after 20 min, the column was equilibrated for further 5 min. The injection volume was 5 µL. Lidocaine hydrochloride (200 µg mL^{-1}) was used as internal standard (IS) and mixed with the samples in a ratio of 1:2 to achieve a final concentration of 100 µg mL^{-1} of IS. Both drugs were detected with a DAD at λ = 220 nm.

2.7 Size distribution and zeta potential measurements. The size distribution of the liposomes was determined by dynamic light scattering. Samples diluted to a concentration of 0.75 mM in ultrapure water were measured at 25 °C using a Litesizer 500 (Anton Paar, Graz, Austria) with a λ = 658 nm laser and 175° backscatter angle in a disposable cuvette (Sarstedt, Nümbrecht, Germany). The zeta potential was determined by means of continuously-monitored phase-analysis light scattering (cmPALS) in an Omega cuvette (Anton Paar, Graz, Austria). The intensity size distribution of the liposomes was typically unimodal; therefore, the autocorrelation function was analyzed according to the cumulant method.

2.8 Photodegradation. Degradation of BUP-loaded liposomes was induced by strong UV radiation exposure and high temperature in a UVA Cube 100 (Dr. Hönle AG, München, Germany) equipped with an iron-radiator and quartz-filter. Samples were exposed to UV radiation (100 W) up to 36 h. After given time points, stability data (lipid and BUP content, hydrodynamic diameter, PDI and zeta potential) were analyzed.

2.9 Statistical evaluation. All measurements were carried out in triplicate and the values are represented as mean ± standard. For results based on calculations with experimental triplicates, the propagated uncertainty of error is displayed.

3. Results and Discussion

3.1 Quantification of phospholipids – Method development and validation. An immediate and reliable quantification, as well as comprehensive stability evaluation for quality control, are of paramount importance in every manufacturing process. With the aim of obtaining an efficient, not MS-coupled profiling strategy for a multitude of phospholipids-based products, we developed a HPLC-DAD-CAD method that would enable a remarkably rapid and simultaneous detection of routine components of (phospho)lipid mixtures and of liposomal components. Differently from other reported methods [36–40], low LODs and LOQs could be obtained injecting very small sample volumes (5 μL). Figure 2 shows overlaid chromatograms of different groups of (phospho)lipids and other liposome-related substances. For a better overview they are separated by headgroup or origin: synthetic phosphatidylcholines (Figure 2a), natural phosphatidyl choline blends SPC and EPC (Figure 2b), sphingomyelin and hydrogenated SPC (Figure 2c), negatively charged phospholipids with phosphatic acid headgroup (Figure 2d), negatively charged phospholipids with phosphatidylglycerol headgroup (Figure 2e), negatively charged phospholipids with phosphatidylserine headgroup (Figure 2f), fatty acids (Figure 2g) and lipophilic fluorescent tracers detected with the DAD (Figure 2h). It can be clearly appreciated that the method enables not only to separate different classes of (phospho)lipids, but also (phospho)lipids differing slightly in their lipid chains (e.g. DPPC, POPC and DOPC (Figure 2a)). A possible mixture of different (phospho)lipids used in the development of drug delivery systems are shown in Figure S1 proving the sensitivity of the method and the broad application possibilities.

Beside the broad area of application, analytical factors like linearity, reproducibility, robustness and accuracy described by the International Conference of Harmonization (ICH) guidelines Q2 (R1) need to be fulfilled. For the evaluation of linearity, three independent stock solutions of each lipid were prepared in

chloroform. For the natural phospholipids and multicomponent mixtures (EPC, SPC, HSPC, ESM,), concentration ranges from 1 mg mL^{-1} (500 µg mL^{-1} for BrainPS) to 1 µg mL^{-1} were used. The stock solutions for (synthetic) pure substances (CHOL, DPPC, DOPC, POPC, DSPC, DPPA, DSPA, DOPA DSPG, DOPG, DPPS, DOPS, POPS, DSPE-mPEG-2000, DPPA-PEG3-mannose) were prepared in a range of 100 µg mL^{-1} (50 µg mL^{-1} for DSPE-mPEG-2000) to 0.5 µg mL^{-1}. The linearity of the lipid-based fluorescent dyes DiO and DiD was assessed between 25 and 0.5 µM representing a commonly used concentration range in liposomal formulations. After analysis, the area under the curve (AUC) of the peak for single components (main peak for multicomponent substances) were analyzed and divided through the AUC of the IS. The given ratio ± standard deviation was plotted against the concentration of the solution. Although CAD detectors are known for their non-linear behavior [41], all the various analysed components show a strong linearity in the chosen formulation-based concentration range given in Table 1. Every obtained linear regression is characterized by a coefficient of determination of $R^2 \geq 0.97$ and a low signal intensity based standard error of regression of $Sy.x \leq 1.3$ for all tested components indicating a strong correlation between the AUC and the initial concentration and fulfilling the criterion of linearity.

The analytical sensitivity of the method was assessed by means of LOD and LOQ as described in the Methods. For the determination of the LOD/LOQ of the natural phospholipids represented by several peaks, the calculations are based on the main peak (peak with highest AUC). Results shown in Table 1 indicate a sufficient LOD and LOQ for all analytes. Using an UHPLC-coupled CAD detector, much lower LOD and LOQ could be achieved in comparison to an HPLC-coupled ELSD detector [42,43]. Compared to other UHPLC methods using the CAD detector, our LOD and LOQ values are in the same range or below [43–46].

The reproducibility of the method was tested by repetition of the calibration curves of DPPC, DOPC and POPC. The results of the respective two calibration curves are shown in Figure S2. All analyzed probes show an excellent correlation and reproducibility.

For the evaluation of accuracy, five independent solutions of three different components (DPPC, DOPC and POPC) with known concentrations were prepared. After the analysis, the concentration of phospholipid was determined and the percentage deviation from the nominal concentration based on the calibration curves was calculated (Table S1). Although there is a trend of increasing deviations by decreasing concentrations of the sample, all samples showed a deviation from the nominal concentration of less than 5% which fulfills the criterion of accuracy.

Modern UHPLC systems constantly monitor all parameters (flow rate, gradient composition, column oven temperature, lamp house temperature of the DAD, gas pressure, ion trap voltage of the CAD, etc.) important for the evaluation of the exactness of the method. As requested by the ICH-Q2A and Q2B guidelines, a robustness test should demonstrate that minor changes of method parameters (flow rate/ column oven temperature/ CAD gas evaporation temperature) or malfunction of intrasystem control mechanisms only minimally affect the precision of the analysis and a method transfer to other machines from other suppliers could be possible without mayor disadvantages following specific transfer protocols [47]. Thanks to modern UHPLC-systems, serious alterations in the underlying conditions lead to an automated shut down of the analysis (in our analytical system, any changes of flow rate or changes in column oven/ CAD gas evaporation temperature of > 0.5 °C). Nevertheless, a physical mixture of three components (DPPC, DOPC, POPC) showing minor distinctions in the retention time were analyzed using altered method parameters to fulfill ICH-Q2A and Q2B guideline's requirements. Changing the temperature results in minor positive (48 °C, +3.41%) and negative (52 °C, -2.66%) variations in the retention time of the eluted samples. No considerable alteration of the AUC could be observed for both parameters, temperature and flow rate (Table S2). Changes in the flow are responsible for expected alterations in retention time slightly increasing with a decreased flow rate. Changes in CAD gas evaporation temperature only had a negligible effect on both flow rate (< 0.3%) and AUC (< 0.6%, Table S2). All components in each sample could be separated and no overlay of the different peaks occurred (**Error! Reference source not found.**3). Thus,

the outcome of the test indicates a strong robustness of the method within the given borders and offers the opportunity for simple method transfer.

3.2 Applicability for quality control of liposomal formulations. The applicability of the UHPLC method for phospholipid quantification was tested with two different formulations, one negatively charged formulation currently used in research for the development of a novel depot drug delivery systems and a second formulation representing the commercially available product Doxil [28,31]. Liposomes were produced by film hydration method (*vide supra*). Briefly, the formed vesicles were subjected to six freeze-thaw cycles and subsequently extruded to achieve a homogenous size distribution. The first formulation, liposomes consisting of DSPG, CHOL and DPPC (25:30:45 mol%), was used for the active encapsulation of BUP. As expected, the formulation resulted homogenously dispersed, with a measured hydrodynamic diameter of 172.8 ± 0.8 nm and a polydispersity index (PDI) of 0.08 ± 0.02. The lipid content was analyzed after every production step. During the preparation and extrusion of the liposomes, no degradations could be observed (Figure 3). The extrusion of the liposomes led to a loss of approximately 7% in the total lipid concentration without inducing changes in relative lipid molar ratios. After liposome formation, BUP was loaded remotely using a transmembrane ammonium gradient formed by means of a buffer-equilibrated SEC column, adapted from previous protocols [34,35]. Afterwards, the unencapsulated BUP was removed by eluting the BUP-containing liposomes through a second SEC column. The sequential SEC elutions induced a slight variation in the final total lipid concentration of the samples of approximately 10%, nevertheless without altering the relative composition of the final formulation. The measured amount of encapsulated BUP was 8.2 ± 0.6 mg mL^{-1} with a final drug to lipid ratio (D/L ratio) of 0.97 ± 0.09.

The Doxil-like vesicles, formulated with HSPC, CHOL and DSPE-mPEG-2000, had a size of 131.9 ± 1.6 nm with a PDI of 0.07 ± 0.02. All the different components could be resolved simultaneously with the described HPLC method as shown in Figure 4**Error! Reference source not found.**a. Furthermore, the quanti-

fication showed that minor loss of lipids (1%) occurred during the preparation and extrusion of the liposomes and the Doxil-like formulation was identical to the theoretical composition of the market product demonstrating the methods suitability for being used in regular quality control (Figure 4b).

3.3 Photodegradation. Previously produced BUP-liposomes were subjected to a stress test to simulate light-induced degradation, possibly responsible of liposome instability during long term storage. After 18 h in the UV chamber, only a moderate degradation of DSPG and DPPC could be detected. However, the CHOL content showed a major decrease from 8.43 ± 0.52 mM to 1.97 ± 2.15 mM (Figure 5) without remarkably changes in the size or zeta potential of the liposomes, only the PDI increased slightly (Figure S4a and b). The BUP amount remained stable at 97.5 ± 4.9%. After 36 h, no CHOL could be detected any longer, while 65% of DSPG and 49% of DPPC were degraded; the BUP amount was reduced to 79.8 ± 4.2%. Based on DLS data, an unequivocal instability was induced (Figure S4a). Contrary to our assumption, no free fatty acids (PA or SA) or other detectable degradation products could be analyzed after the photodegradation. This might origin from the unnaturally strong energy input of the UV-chamber and a complete degradation/oxidation of the phospholipids towards monomolecular products without the intermediate step of hydrolyzation [48]. The decrease of zeta potential of around 10 mV indicates the formation of negatively charged degradation products (Figure S4b).

4. Conclusion

The described CAD-based UHPLC method offers the opportunity of analyzing simultaneously the composition and stability of differently charged phospholipids, lipids, and active pharmaceutical ingredients used in commercially available formulations and in research. We could demonstrate that all the requirements given by the ICH-Q2A and Q2B guidelines are fulfilled. The method shows a strong linearity for a broad multitude of chemically different analytes used in liposomal formulations. Furthermore,

a sufficient LOD/LOQ associated with an ideal reproducibility was achieved for all probes. Finally, our approach combines a comprehensive and efficient quantification with minimal economic and ecologic impact. The herein proposed method meets the requirements of modern analytical laboratories, offering a broad range of possible applications in either quality control or process observation in various industrial sectors and scientific laboratories.

Supporting Information

Chromatogram of a possible mixture of various (phospho)lipids, validation of the method (reproducibility, robustness, accuracy), size and zeta potential changes of photodegradation test (PDF).

Corresponding Author

*Paola Luciani – Pharmaceutical Technology Laboratory, Department of Chemistry and Biochemistry, University of Bern, Switzerland; Phone: +41 31 631 41 60; Email: paola.luciani@dcb.unibe.ch

Acknowledgement

The Phospholipid Research Center is kindly acknowledged for the financial support (P.L.).

References

[1] E. Beltrán-Gracia, A. López-Camacho, I. Higuera-Ciapara, J.B. Velázquez-Fernández, A.A. Vallejo-Cardona, Nanomedicine review: Clinical developments in liposomal applications, Springer Vienna, 2019. https://doi.org/10.1186/s12645-019-0055-y.

[2] R.P. Singh, H. V. Gangadharappa, K. Mruthunjaya, Phospholipids: Unique carriers for drug delivery

systems, J. Drug Deliv. Sci. Technol. 39 (2017) 166–179. https://doi.org/10.1016/j.jddst.2017.03.027.

[3] GRAS EXEMPTION CLAIM for SunPS Manufactured by ECA Healthcare, Inc., (2016).

[4] P. van Hoogevest, Review – An update on the use of oral phospholipid excipients, Eur. J. Pharm. Sci. 108 (2017) 1–12. https://doi.org/10.1016/j.ejps.2017.07.008.

[5] P. van Hoogevest, A. Fahr, Phospholipids in Cosmetic Carriers, in: Nanocosmetics, Springer International Publishing, Cham, 2019: pp. 95–140. https://doi.org/10.1007/978-3-030-16573-4_6.

[6] L. Cui, E.A. Decker, Phospholipids in foods: Prooxidants or antioxidants?, J. Sci. Food Agric. 96 (2016) 18–31. https://doi.org/10.1002/jsfa.7320.

[7] I. Santiago, F.C. Simmel, Self-Propulsion Strategies for Artificial Cell-Like Compartments, Nanomaterials. 9 (2019) 1680. https://doi.org/10.3390/nano9121680.

[8] C. Isalomboto Nkanga, A. Murhimalika Bapolisi, N. Ikemefuna Okafor, R. Werner Maçedo Krause, General Perception of Liposomes: Formation, Manufacturing and Applications, in: Liposomes - Adv. Perspect., IntechOpen, 2019. https://doi.org/10.5772/intechopen.84255.

[9] D.J.A. Crommelin, P. van Hoogevest, G. Storm, The role of liposomes in clinical nanomedicine development. What now? Now what?, J. Control. Release. 318 (2020) 256–263. https://doi.org/10.1016/j.jconrel.2019.12.023.

[10] S. Ait-Oudhia, D.E. Mager, R.M. Straubinger, Application of pharmacokinetic and pharmacodynamic analysis to the development of liposomal formulations for oncology, Pharmaceutics. 6 (2014) 137–174. https://doi.org/10.3390/pharmaceutics6010137.

[11] G. Bastiat, C.O. Pritz, C. Roider, F. Fouchet, E. Lignières, A. Jesacher, R. Glueckert, M. Ritsch-Marte,

A. Schrott-Fischer, P. Saulnier, J.P. Benoit, A new tool to ensure the fluorescent dye labeling stability of nanocarriers: A real challenge for fluorescence imaging, J. Control. Release. 170 (2013) 334–342. https://doi.org/10.1016/j.jconrel.2013.06.014.

[12] G.R. Bartlett, Phosphorus assay in column chromatography., J. Biol. Chem. 234 (1959) 466–468. https://doi.org/10.1111/j.1365-2621.1976.tb01121.x.

[13] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14. https://doi.org/10.1016/0003-2697(80)90269-9.

[14] L. Ferey, S.A. Slabi, C.E. Roy, P. Barthelemy, K. Gaudin, Chromatographic study of nucleoside-lipids by RP-UHPLC-DAD/CAD, Anal. Bioanal. Chem. 410 (2018) 7711–7721. https://doi.org/10.1007/s00216-018-1388-9.

[15] K. Schilling, U. Holzgrabe, Recent applications of the Charged Aerosol Detector for liquid chromatography in drug quality control, J. Chromatogr. A. (2020) 460911. https://doi.org/10.1016/j.chroma.2020.460911.

[16] M. Doktorova, F.A. Heberle, B. Eicher, R.F. Standaert, J. Katsaras, E. London, G. Pabst, D. Marquardt, Preparation of asymmetric phospholipid vesicles : The next generation of cell membrane models, Nat Protoc. 13 (2019) 2086–2101. https://doi.org/10.1038/s41596-018-0033-6.Preparation.

[17] M. Cansell, T. Bardeau, E. Morvan, A. Grélard, C. Buré, P. Subra-Paternault, Phospholipid Profiles of Oleaginous Pressed Cakes Using NMR and Gas Chromatography, JAOCS, J. Am. Oil Chem. Soc. 94 (2017) 1219–1223. https://doi.org/10.1007/s11746-017-3022-y.

[18] L. Ferey, A. Raimbault, I. Rivals, K. Gaudin, UHPLC method for multiproduct pharmaceutical analysis by Quality-by-Design, J. Pharm. Biomed. Anal. 148 (2018) 361–368.

https://doi.org/10.1016/j.jpba.2017.10.014.

[19] F.B. Jungalwala, R.J. Turel, J.E. Evans, R.H. McCluer, Sensitive analysis of ethanolamine and serine containing phosphoglycerides by high performance liquid chromatography, Biochem. J. 145 (1975) 517–526. https://doi.org/10.1042/bj1450517.

[20] Y.H. Rustam, G.E. Reid, Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics, Anal. Chem. 90 (2018) 374–397. https://doi.org/10.1021/acs.analchem.7b04836.

[21] R.W. Dixon, D.S. Peterson, Development and testing of a detection method for liquid chromatography based on aerosol charging, Anal. Chem. 74 (2002) 2930–2937. https://doi.org/10.1021/ac011208l.

[22] J.M. Charlesworth, Evaporative Analyzer as a Mass Detector for Liquid Chromatography, Anal. Chem. 50 (1978) 1414–1420. https://doi.org/10.1021/ac50033a011.

[23] W.S.M. Geurts van Kessel, M. Tieman, R.A. Demel, Purification of phospholipids by preparative high pressure liquid chromatography, Lipids. 16 (1981) 58–63. https://doi.org/10.1007/BF02534922.

[24] A.I. Hopia, V.M. Olialainen, Comparison of the evaporative light scattering detector (ELSD) and refractive index detector (RID) in lipid analysis, J. Liq. Chromatogr. 16 (1993) 2469–2482. https://doi.org/10.1080/10826079308019586.

[25] T. Wang, D. Zhou, Advances in phospholipid quantification methods, Curr. Opin. Food Sci. 16 (2017) 15–20. https://doi.org/10.1016/j.cofs.2017.06.007.

[26] B.L. Peterson, B.S. Cummings, A review of chromatographic methods for the assessment of phospholipids in biological samples, Biomed. Chromatogr. 20 (2006) 227–243. https://doi.org/10.1002/bmc.563.

[27] M.W. Robinson, A.P. Hill, S.A. Readshaw, J.C. Hollerton, R.J. Upton, S.M. Lynn, S.C. Besley, B.J. Boughtflower, Use of Calculated Physicochemical Properties to Enhance Quantitative Response When Using Charged Aerosol Detection, Anal. Chem. 89 (2017) 1772–1777. https://doi.org/10.1021/acs.analchem.6b04060.

[28] L. Rahnfeld, J. Thamm, F. Steiniger, P. van Hoogevest, P. Luciani, Study on the in situ aggregation of liposomes with negatively charged phospholipids for use as injectable depot formulation, Colloids Surfaces B Biointerfaces. 168 (2018) 10–17. https://doi.org/10.1016/j.colsurfb.2018.02.023.

[29] U. S. Food and Drug Administration, Guideline for Industry: Text on Validation of Analytical Procedures (ICH-Q2A), Food Drug Adm. (1995) 1–9. https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm073381.pdf.

[30] U. S. Food and Drug Administration, Guidance for Industry Q2B Validation of Analytical Procedures: Methodology, 20857 (1996) 301–827. http://www.fda.gov/cder/guidance/index.htm%5Cnhttp://www.fda.gov/cber/guidelines.htm.

[31] Y. Barenholz, Doxil - The first FDA-approved nano-drug: Lessons learned, J. Control. Release. 160 (2012) 117–134. https://doi.org/10.1016/j.jconrel.2012.03.020.

[32] M. Hadjidemetriou, Z. Al-Ahmady, K. Kostarelos, Time-evolution of in vivo protein corona onto blood-circulating PEGylated liposomal doxorubicin (DOXIL) nanoparticles, Nanoscale. 8 (2016) 6948–6957. https://doi.org/10.1039/C5NR09158F.

[33] M.J.J. Hope, M.B.B. Bally, G. Webb, P.R.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, BBA - Biomembr. 812 (1985) 55–65.

https://doi.org/10.1016/0005-2736(85)90521-8.

[34] R. Cohen, A. Steiner, H. Kanaan, Y. Barenholz, Chemical and physical characterization of remotely loaded bupivacaine liposomes: comparison between large multivesicular vesicles and small unilamellar vesicles, J. Mater. Chem. B. 1 (2013) 4619–4627. https://doi.org/10.1039/c3tb20609b.

[35] G.J. Grant, Y. Barenholz, E.M. Bolotin, M. Bansinath, H. Turndorf, B. Piskoun, E.M. Davidson, M. Bansinath, G.J. Grant, E.M. Davidson, E.M. Bolotin, Y. Barenholz, H. Turndorf, A novel liposomal bupivacaine formulation to produce ultralong-acting analgesia, Anesthesiology. 101 (2004) 133–137. https://doi.org/10.1097/01.sa.0000151238.77039.f4.

[36] D. Van Haute, W. Jiang, T. Mudalige, Evaluation of size-based distribution of drug and excipient in amphotericin B liposomal formulation, Int. J. Pharm. 569 (2019) 118603. https://doi.org/10.1016/j.ijpharm.2019.118603.

[37] G. Kiełbowicz, P. Micek, C. Wawrzeńczyk, A new liquid chromatography method with charge aerosol detector (CAD) for the determination of phospholipid classes. Application to milk phospholipids, Talanta. 105 (2013) 28–33. https://doi.org/10.1016/j.talanta.2012.11.051.

[38] C. Schönherr, S. Touchene, G. Wilser, R. Peschka-Süss, G. Francese, Simple and precise detection of lipid compounds present within liposomal formulations using a charged aerosol detector, J. Chromatogr. A. 1216 (2009) 781–786. https://doi.org/10.1016/j.chroma.2008.11.080.

[39] H. Shibata, C. Yomota, H. Okuda, Simultaneous determination of polyethylene glycol-conjugated liposome components by using reversed-phase high-performance liquid chromatography with UV and evaporative light scattering detection, AAPS PharmSciTech. 14 (2013) 811–817. https://doi.org/10.1208/s12249-013-9967-8.

[40] D. Jeschek, G. Lhota, J. Wallner, K. Vorauer-Uhl, A versatile, quantitative analytical method for pharmaceutical relevant lipids in drug delivery systems, J. Pharm. Biomed. Anal. 119 (2016) 37–44. https://doi.org/10.1016/j.jpba.2015.11.020.

[41] L.E. Magnusson, D.S. Risley, J.A. Koropchak, Aerosol-based detectors for liquid chromatography, J. Chromatogr. A. 1421 (2015) 68–81. https://doi.org/10.1016/j.chroma.2015.07.045.

[42] C. Roces, E. Kastner, P. Stone, D. Lowry, Y. Perrie, Rapid Quantification and Validation of Lipid Concentrations within Liposomes, Pharmaceutics. 8 (2016). https://doi.org/10.3390/pharmaceutics8030029.

[43] R.G. Ramos, D. Libong, M. Rakotomanga, K. Gaudin, P.M. Loiseau, P. Chaminade, Comparison between charged aerosol detection and light scattering detection for the analysis of Leishmania membrane phospholipids, J. Chromatogr. A. 1209 (2008) 88–94. https://doi.org/10.1016/j.chroma.2008.07.080.

[44] J. Damnjanović, H. Nakano, Y. Iwasaki, Simple and efficient profiling of phospholipids in phospholipase D-modified Soy lecithin by HPLC with charged aerosol detection, JAOCS, J. Am. Oil Chem. Soc. 90 (2013) 951–957. https://doi.org/10.1007/s11746-013-2236-x.

[45] K.M. Barry, T.G. Dinan, B.A. Murray, P.M. Kelly, Comparison of dairy phospholipid preparative extraction protocols in combination with analysis by high performance liquid chromatography coupled to a charged aerosol detector, Int. Dairy J. 56 (2016) 179–185. https://doi.org/10.1016/j.idairyj.2016.01.022.

[46] V. Agostoni, S.H. Lee, V. Forster, M. Kabbaj, C.R. Bosoi, M. Tremblay, M. Zadory, C.F. Rose, J.C. Leroux, Liposome-Supported Peritoneal Dialysis for the Treatment of Hyperammonemia-Associated Encephalopathy, Adv. Funct. Mater. 26 (2016) 8382–8389.

https://doi.org/10.1002/adfm.201603519.

[47] FDA, Protocols for the Conduct of Method Transfer Studies for Type C Medicated Feed Assasy Methods, 2007. http://www.fda.gov/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/default.htm. (accessed April 8, 2020).

[48] K. Parchem, S. Sasson, C. Ferreri, A. Bartoszek, Qualitative analysis of phospholipids and their oxidised derivatives–used techniques and examples of their applications related to lipidomic research and food analysis, Free Radic. Res. 53 (2019) 1068–1100. https://doi.org/10.1080/10715762.2019.1657573.

Figures

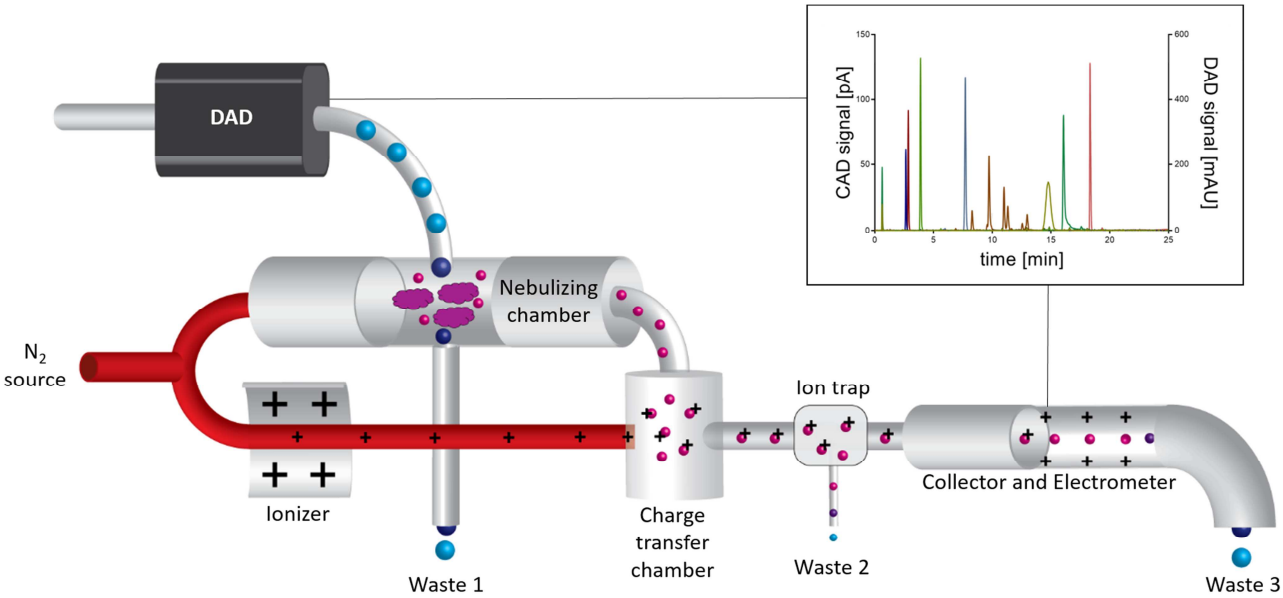


Figure 1. Schematic overview of a typical CAD setup.

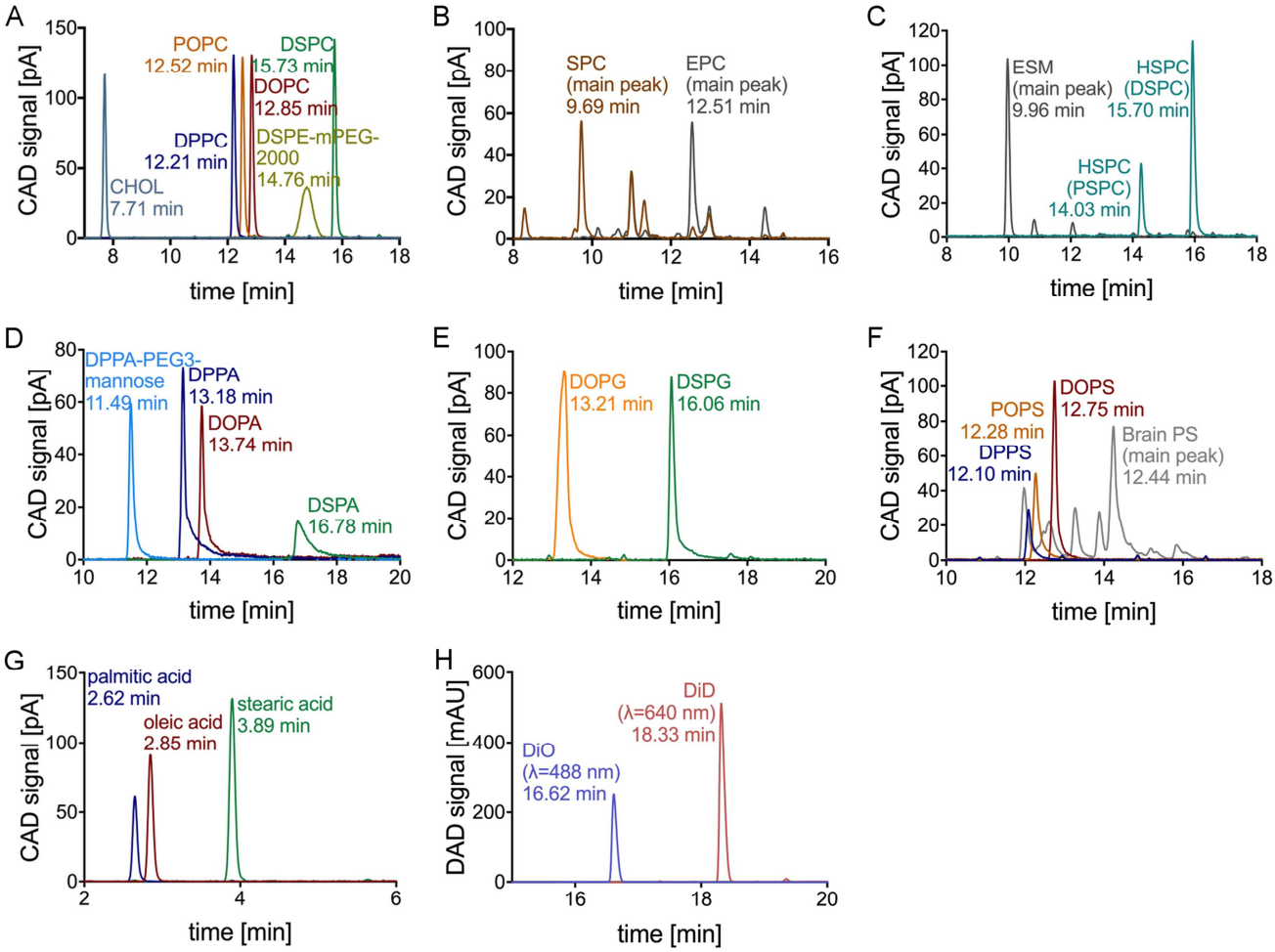


Figure 2. HPLC chromatograms of different phospholipids, (a) synthetic, amphiphilic phospholipids with phosphatidylcholine headgroup, CHOL and DSPE-PEG, (b) natural, amphiphilic phospholipids EPC and SPC, (c) ESM and hydrogenated SPC (HSPC), (d) negatively charged phospholipids with phosphatic acid headgroup, (e) negatively charged phospholipids with phosphatidyl glycerol headgroup, (f) negatively charged phospholipids with phosphatidylserine headgroup, (g) fatty acids and (h) the lipophilic fluorescent tracers DiO and DiD detected with the DAD.

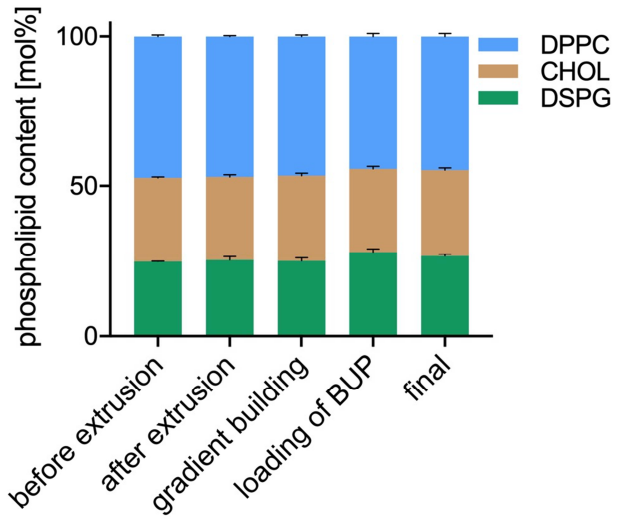


Figure 3. Composition of liposome formulations containing 25 mol% DSPG, 30 mol% CHOL and 45 mol% DPPC during the preparation and the encapsulation of BUP with a final D/L ratio of 0.97 ± 0.09.

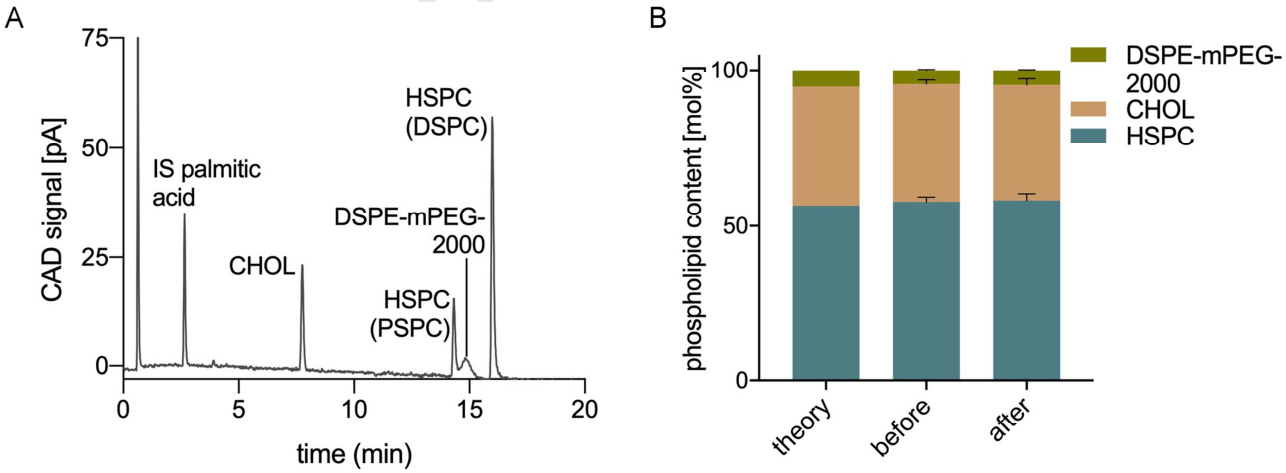


Figure 4. (a) Chromatogram of Doxil-like liposomes containing HSPC, CHOL and DSPE-mPEG-2000. (b) Theoretical content (molar ratio) of Doxil and composition of Doxil-like liposomes before and after extrusion.

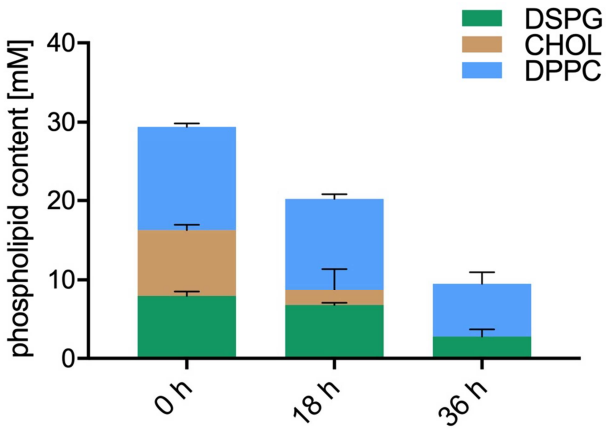


Figure 5. Photodegradation of BUP liposomes. Molar concentrations of DSPG, CHOL and DPPC before and after 18 h and 36 h exposure time.

Tables

Table 1. Analyzed phospholipids with their respective retention time, the linearity range, coefficient of determination (R^2), standard error of regression (Sy.x) and limit of detection (LOD) and limit of quantification (LOQ). For mixtures of natural phospholipids, the retention time of the main peak and the corresponding LOD and LOQ are displayed.

Phospholipid	Detection	Retention time [min]	Linearity range [µg mL^{-1}]		R^2	Sy.x	LOD [µg mL^{-1}]	LOQ [µg mL^{-1}]
			min	max				
DPPC	CAD	12.21	0.5	100	0.9919	0.1441	1.0	5.0
DSPC	CAD	15.73	0.5	100	0.9906	0.1615	0.5	2.5
DOPC	CAD	12.85	0.5	100	0.9826	0.2050	0.5	2.5
POPC	CAD	12.52	0.5	100	0.9947	0.1042	0.1	2.5
EPC	CAD	12.51	1.0	1000	0.9731	0.7236	1.0	10
SPC	CAD	9.69	5.0	1000	0.9844	0.5525	10	25
HSPC	CAD	15.70	5.0	1000	0.9829	1.2910	2.5	25
DPPA	CAD	13.18	2.5	100	0.9855	0.2334	1.0	2.5
DPPA-PEG3-mannose	CAD	11.49	2.5	100	0.9989	0.0446	2.5	10
DSPA	CAD	16.78	2.5	100	0.9889	0.1810	5.0	25
DOPA	CAD	13.74	2.5	100	0.9747	0.2255	1.0	25
DSPG	CAD	16.06	0.5	100	0.9843	0.2310	1.0	5.0
DOPG	CAD	13.21	0.5	100	0.9848	1.2360	0.5	1.0
DPPS	CAD	12.09	1.0	100	0.9827	0.2822	1.0	2.5
DOPS	CAD	12.75	0.5	100	0.9838	0.1680	1.0	10
POPS	CAD	12.10	1.0	100	0.9764	0.3072	2.5	10
Brain PS	CAD	12.44	10	500	0.9753	0.7449	10	25
ESM	CAD	9.96	1.0	1000	0.9904	0.9270	1.0	5.0
DSPE-mPEG-2000	CAD	14.76	0.5	50	0.9806	0.1076	0.5	25
Cholesterol	CAD	7.71	0.5	100	0.9902	0.1367	0.5	2.5
DiO	DAD	16.62	0.02	22	0.9998	0.0689	< 0.02	< 0.02
DiD	DAD	18.33	0.03	26	0.9988	0.3436	< 0.03	< 0.03

Highlights

Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control

Florian Weber[‡], Lisa Rahnfeld[‡], Paola Luciani*

Pharmaceutical Technology Laboratory, Department of Chemistry and Biochemistry, University of Bern, Switzerland

[‡] contributed equally

* corresponding author

- Rapid UHPLC-CAD method for qualitative and quantitative analysis of phospholipids
- Separation of phospholipids varying in head group and acyl chain
- Simultaneous detection of phospholipids and lipophilic fluorescent markers
- Stability testing and observation of degradation and hydrolyzation
- Confirmed performance by the analysis of two liposomal formulations

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Supporting information

Analytical profiling and stability evaluation of liposomal drug delivery systems: a rapid UHPLC-CAD-based approach for phospholipids in research and quality control

Florian Weber‡, Lisa Rahnfeld‡, Paola Luciani*

Pharmaceutical Technology Research Group, Department of Chemistry and Biochemistry, University of Bern, Switzerland

‡ contributed equally

* corresponding author

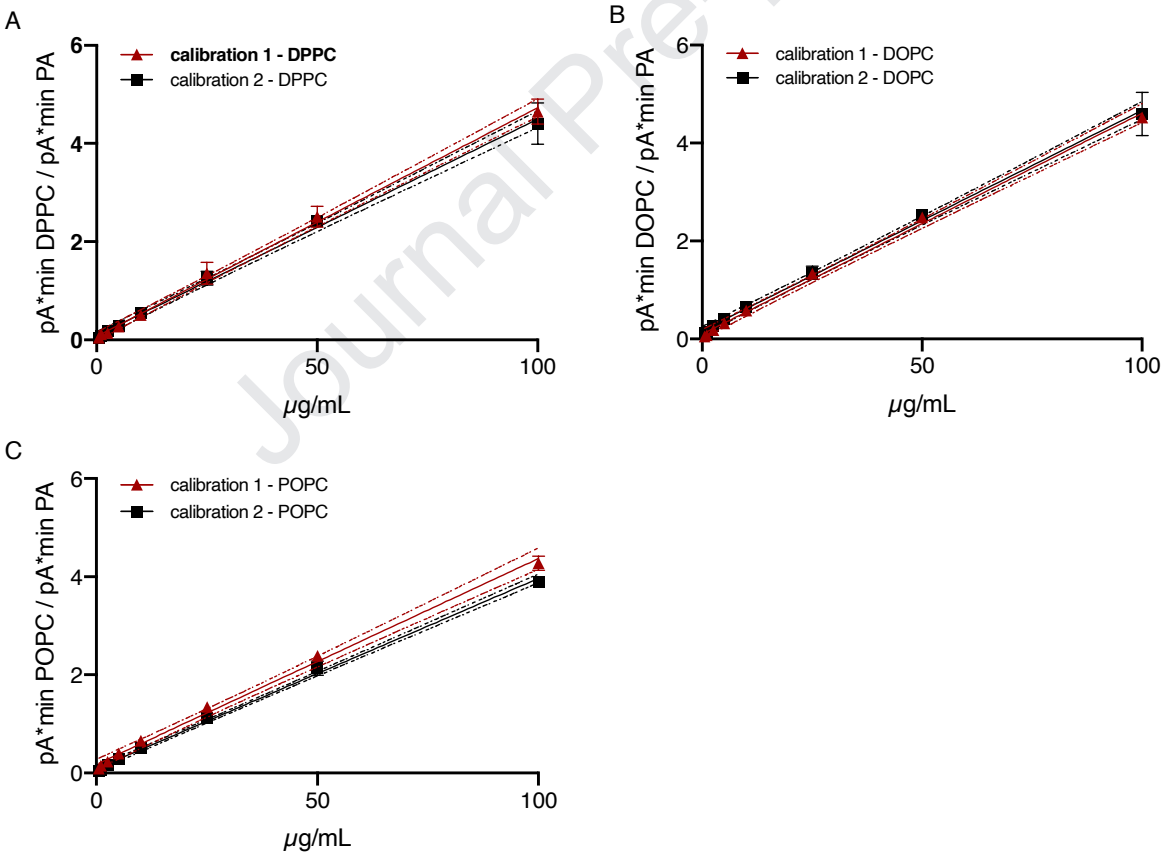


Figure S1. Reproducibility of the HPLC method shown for three different phospholipids, (a) DPPC, (b) DOPC and (c) POPC. Single points on the calibration curve are shown as mean ± SD (n=3). A regression line by the method of least squares is plotted with the 95% confidence interval.

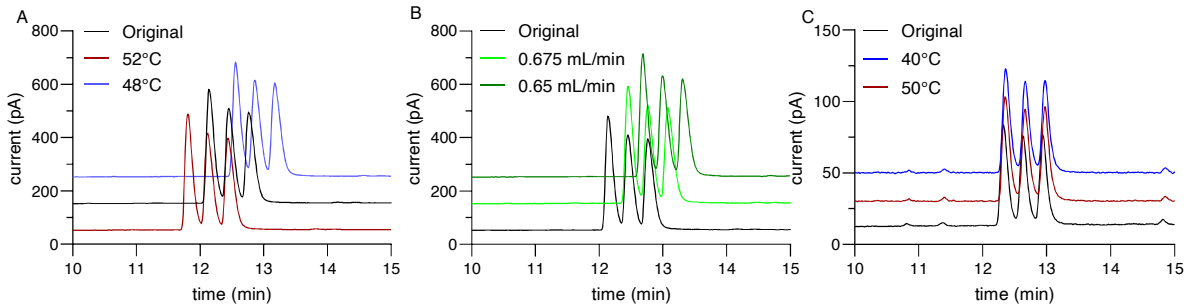


Figure S2. Robustness test of the HPLC method: overlaid chromatograms of mixed, DPPC, DOPC and POPC after being analyzed in altered conditions, (a) column oven temperature, (b) flow rate, (c) CAD gas evaporation temperature.

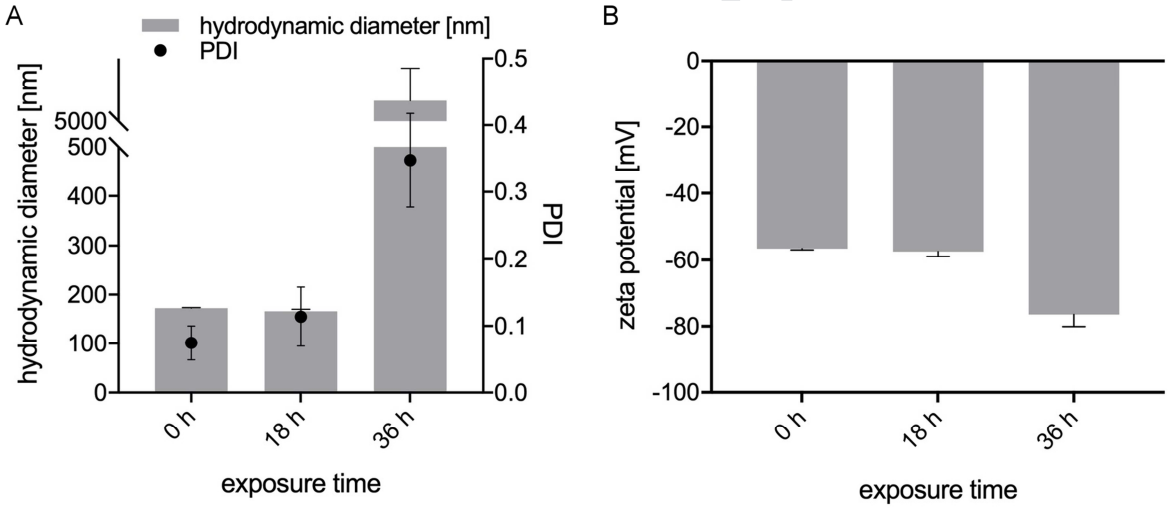


Figure S3. Changes in (a) size and PDI and (b) zeta potential of liposomal formulation after photodegradation.

Table S1. Overview of results from the accuracy test. Deviation of determined concentration of DPPC, DOPC and POPC at different levels from the nominal concentration (n = 5).

Sample concentration	Deviation from nominal concentration [%]		
	DPPC	DOPC	POPC
100 μg/mL	2.35 ± 1.02	2.14 ± 1.13	3.90 ± 1.16
50 μg/mL	5.66 ± 1.39	5.15 ± 1.15	1.42 ± 1.08
25 μg/mL	1.53 ± 1.24	3.42 ± 1.59	1.79 ± 0.61

Table S2. Overview of results obtained by robustness test. Deviation of determined concentrations of combined AUC of DPPC, DOPC and POPC from the nominal concentration after the analysis in altered conditions.

Alteration	Deviation from the nominal value [%]	
	AUC	Retention time
Flow rate: 0.675 mL/min	5.51 ± 1.27	2.51 ± 0.04
Flow rate: 0.65 mL/min	5.88 ± 0.94	4.33 ± 0.09
Column oven: 48 °C	1.60 ± 1.04	3.41 ± 0.10
Column oven: 52 °C	2.15 ± 1.04	2.66 ± 0.05
CAD: 40 °C	0.26 ± 0.04	0.24 ± 0.04
CAD: 50 °C	0.55 ± 0.02	0.20 ± 0.08